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Original article

Melatonin alterations and brain acetylcholine lesions in sleep disorders in Cockayne syndrome

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Abstract

Background: Cockayne syndrome (CS) is a genetic disorder caused by deficient nucleotide excision repair. Patients with CS exhibit progeroid features, developmental delay, and various neurological disorders; they are also known to suffer from sleep problems, which have never been investigated in detail. *Objective:* The aim of this study is to investigate the pathogenesis of sleep disorders in patients with CS. *Methods:* We performed a questionnaire survey of the families of patients with CS, enzyme-linked immunosorbent analyses of the melatonin metabolite, 6-sulphatoxymelatonin (6-SM), in the patients' urine, and immunohistochemistry in the hypothalamus, the basal nucleus of Meynert (NbM), and the pedunculopontine tegmental nucleus (PPN) in four autopsy cases. *Results:* Sleep–wakefulness rhythms were disturbed in patients with CS, and these disturbances seemed to be related to a reduced urinary excretion of 6-SM. In addition, although the hypothalamic nuclei were comparatively preserved, acetylcholine neurons (AchNs) were severely decreased in the NbM and PPN. *Conclusions:* AchNs modulate both arousal and rapid eye movement sleep, and selective lesions of AchNs in the PPN and/or NbM in combination with disturbed melatonin metabolism might be involved in the sleep disorders in CS.
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Keywords: Cockayne syndrome; Sleep disorders; Melatonin; Immunohistochemistry; Hypothalamus; Acetylcholine

1. Introduction

Cockayne syndrome (CS) is a rare genetic disorder that is caused by deficient nucleotide excision repair [1], and postnatal growth failure with a loss of fat; psychomotor developmental delays become evident in patients with CS in infancy or early childhood. Affected children have characteristic facial features, such as sunken eyes, sharp noses, and carious teeth, and they sunburn easily [2]. In addition to developmental delay, patients with CS suffer from neuropathy, visual impairments, neural deafness, cerebellar ataxia, and spasticity, all of which interfere with their quality of life. Autopsies of these patients have demonstrated various pathological changes throughout the peripheral and central nervous systems, including demyelinating peripheral neuropathy, pigmentary retinopathy, optic atrophy, a small brain, patchy demyelination and atrophy of the white matter, calcification predominating in the basal ganglia, and cerebellar atrophy with neuronal loss and gliosis [3,4]. Nancy and Berry [5] have divided 140 published CS cases into the following three types: type I, which is the most prevalent classical childhood disorder; type II, which is the severe congenital or infantile variant of the disorder; and type III, which has an atypical

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late onset of the disorder with prolonged survival. CS patients in Japan are known to exhibit sleep abnormalities and thermoregulation problems, which have rarely been examined in detail. We conducted a preliminary enzyme-linked immunosorbent analysis (ELISA) of the urinary secretion of melatonin metabolites and found a possible impairment of melatonin metabolism in patients with severe motor and intellectual disabilities [6]. In addition, we found a selective reduction of acetylcholine neurons (AchNs) in the basal nucleus of Meynert (NbM) and the pedunculopontine tegmental nucleus (PPN) in autopsy cases with xeroderma pigmentosum group A (XP-A), which is also caused by a hereditary nucleotide excision repair deficiency and which results in cutaneous photosensitivity and various neurodegenerative symptoms but not severe sleep problems [7].

In order to investigate the pathogenesis of sleep disorders in patients with CS, we performed a questionnaire survey in the families of patients with CS, a comprehensive ELISA measurement of 6-sulphatoxymelatonin (6-SM), which is a predominant melatonin metabolite, in the patients' urine, and immunohistochemistry in the hypothalamus, NbM, and PPN in four autopsy cases. Sleep problems were observed in patients with CS, and the disturbed metabolism of melatonin and/or the selective reduction of AchNs may be involved in sleep disorders.

2. Materials and methods

2.1. Questionnaire survey

We made a paper-and-pencil questionnaire that was specific for CS. In collaboration with the Japan Cockayne Syndrome Network, the questionnaires were distributed to family members of patients in the network. The questionnaire was written in Japanese and contained 13 questions. It assessed the current motor and mental abilities, presence or absence of auditory and visual impairments, bedtimes and rising times, naps, the use of hypnotics, and the presence or absence of sleep problems and thermal dysregulation in the patients.

2.2. Measurement of 6-SM and data analysis

Melatonin has a 24-h rhythm that peaks during the night in normally entrained individuals. This has been examined with the plasma and saliva levels of melatonin *per se* or the urinary secretion of 6-SM [8]. Urine samples were collected in the morning, which is when the urinary 6-SM secretion shows its maximum level, and these samples thus reflect the peak melatonin production during the previous night [9]. We measured the urinary excretion of 6-SM early in the morning in 11 samples from nine patients with CS aged from 4 to 31 years (4 males and 7 females), eight samples of eight patients with XP-A with ages from 7 to 29 years (5 males and 3 females), and nine samples of nine age-matched controls who did not have any chronic disorders with ages from 4 to 32 years (4 males and 5 females). The analysis of the urine 6-SM was performed with an ELISA, using an assay kit from GenWay Biotech, Inc. (San Diego, CA, USA). The urine samples were diluted prior to the assay. The results were revised by the creatinine (Cre) values, and we obtained a corrected urinary value of 6-SM (ng/mg Cre).

All of the data are presented as the mean [standard deviation (SD)]. The Stat Flex statistical program, version 6 (Artech Co., Ltd., Osaka, Japan), was used for the data analysis [10]. Bartlett's test was used to confirm whether the samples had equal variances, and they were judged to have inhomogeneous variances. Independent samples were examined by a Kruskal–Wallis test in order to compare the nonparametric data among controls, patients with CS, and patients with XP-A. A Mann–Whitney *U*-test was used to compare the nonparametric variables between each pair of groups.

2.3. Immunohistochemical analyses of the autopsy brains

The subjects included four cases of clinically and genetically confirmed CS who were aged from 7 to 35 years and six controls who did not have any pathological changes in the central nervous system and who were aged from 4 months to 38 years. Coronal sections of each formalin-fixed brain sample were cut and embedded in paraffin. Serial 6-μm-thick sections were cut from the diencephalon, including the hypothalamus and the NbM, and the lower midbrain, including the PPN. After microwave antigen retrieval, each section was treated with mouse monoclonal antibodies to microtubule-associated protein 2 (MAP2; EMD Millipore Corporation, Billerica, MA, USA), glial acidic fibrillary protein (GFAP; Nichirei Biosciences Inc., Tokyo, Japan), acetylcholinesterase (AchE; Thermo Fisher Scientific Inc., Rockford, IL, USA), tyrosine hydroxylase (TH; Thermo Fisher Scientific Inc.), calbindin-D28K (CD; Leica Microsystems Ltd., Milton Keynes, UK), vasopressin (VP; Biomeda Corporation, Foster City, CA, USA), and orexin A (OxA; Nuclea Diagnostic Laboratories LLC, Cambridge, MA, USA) at the following concentrations: 1:1 (GFAP), 1:100 (MAP2, CD, VP, and OxA), 1:250 (AchE), and 1:400 (TH). Antibody binding was visualized with the avidin–biotin–immunoperoxidase complex method (Nichirei Biosciences Inc.) according to the manufacturer's protocol. No staining was confirmed in the sections in the absence of antibody.

2.4. Quantitative evaluation and data analysis

The location of NbM was determined by immunohistochemistry for CD and was ventral to the globus

pallidus, and neurons immunoreactive for AchE were counted. The PPN was identified as dorsolateral to the rostral superior cerebellar peduncle and the medial lemniscus in the lower midbrain, as previously reported [7]. The PPN is composed of clusters of moderately large neurons (pars compacta) and the more widespread pars dissipata in the rostral and medial regions. In the pars compacta of the PPN, the number of neurons that were immunoreactive for MAP2, AchE, TH, and CD were determined after the manual labeling of appropriate neurons with nucleoli in two serial sections, and the mean values were calculated. The percentages of neurons that were immunoreactive for AchE, TH, and CD relative to those that were immunoreactive for MAP2 were calculated. All data are presented as the mean (SD), and the Stat Flex statistical program, version 6, was used for the data analysis. Mann–Whitney's *U*-test was used to compare the nonparametric data between CS cases and controls for a quantitative evaluation of the immunoreactive cells.

2.5. Ethical aspects

Informed consent was provided by the patients' families, who participated in the questionnaire survey, for the measurement of urinary secretion of 6-SM, and the postmortem research using autopsy brains. The ethical committee of the Tokyo Metropolitan Institute of Medical Science approved all of the parts of this study.

3. Results

3.1. Questionnaire survey

Answers were obtained from 10 patients, and the data are summarized in Table 1. All patients were mobile. Nine of the 10 patients, except for patient 4, had deafness, and all patients showed visual disabilities due to cataracts and/or retinal pigmentation. Six patients had sleep problems, such as daytime drowsiness and frequent arousal or excitement during sleep. Bedtimes and/or rising times showed an abnormal shift in patients 3 and 7, indicating the possibility of circadian rhythm disorders. Patient 8 had a history of taking hypnotics, which had no beneficial effects (data not shown). Thermal regulation was disturbed in seven patients, five of whom demonstrated poor peripheral circulation. Patients 2, 3, 7, and 8 had disturbances in both sleep and thermal regulation.

3.2. Measurement of urinary secretion of 6-SM

The means (SD) of the values of the urinary 6-SM secretion were 103.2 (83.4) ng/mg Cre in controls, 15.7 (8.5) ng/mg Cre in CS patients, and 56.7 (89.6) ng/mg Cre in XP-A patients, respectively, indicating a significant difference among the three groups as confirmed by a Kruskal–Wallis test ($P = 0.001279$). A significant decrease in urinary 6-SM secretion was found in the CS patients versus the controls ($P < 0.001$). The urinary

Table 1
Summary of the questionnaire survey.

	Age/sex	Motor ability	Sensory impairments				Nap (/day)	Sleep problems	Thermal dysregulation	Disturbed peripheral circulation
			Auditory	Visual	Rising time	Bed-time				
1	4 yrs/male	Walk with support	1+	1+	6:00–7:00	19:00–21:00	1	(−)	(−)	(−)
2	5 yrs/female	Walk with support	2+	1+	6:00–7:00	20:00–21:00	1	Daytime drowsiness	Hypothermia	1+
3	6 yrs/female	Walk with support	2+	1+	7:00–10:00	20:00–21:00	1–2	Hard to rise	Frequent fever	(−)
4	7 yrs/male	Walk with support	(−)	1+	6:00–7:00	21:00–22:00	1	(−)	Poikilothermia	1+
5	9 yrs/male	Creep	2+	1+	6:00–7:00	21:00–22:00	1	(−)	Poikilothermia	1+
6	10 yrs/female	Creep	2+	1+	7:00–8:00	23:00–0:00	1	(−)	Hypothermia	1+
7	12 yrs/female	Sit	2+	1+	4:00–9:00	23:00–3:00	1	Hard to sleep and rise	Fever responding to environment	1+
8	21 yrs/male	Wheel chair	2+	1+	6:00–8:00	20:00–22:00	0–1	Arousal during sleep	Fever responding to environment	(−)
9	31 yrs/female	Wheel chair	1+	1+	7:00–8:00	19:00–20:00	0–1	Excitement in sleep	(−)	(−)
10	31 yrs/female	Wheel chair	1+	1+	7:00–8:00	19:00–20:00	1	Excitement in sleep	(−)	(−)

In auditory impairment, 1+ and 2+ denote "audible with a hearing aid" and "completely deaf," respectively.

6-SM secretion in XP-A patients also seemed to be decreased (Fig. 1), but, when compared with that of controls, the change was not significant because of a high variance in the values.

3.3. Research in autopsy brains

Regardless of the presence or absence of sleep problems and/or thermal dysregulation, all CS cases demonstrated neurons that were immunoreactive for TH in the supraoptic, paraventricular, and ventromedial nuclei in the hypothalamus (Table 2 and Fig. 2A). In addition, two and three cases each showed neurons that were immunoreactive for VP and OxA in the lateral area and ventromedial nucleus in the hypothalamus, respectively (Table 2 and Fig. 2B). Sections that included either the suprachiasmatic nucleus or the pineal gland were not obtained in the CS cases due to the severe brain atrophy. In the NbM, the mean (SD) of the total number of AchNs that were immunoreactive for AchE was 91.7 (25.7) in controls, and they were completely absent in CS cases, indicating a significant loss of AchNs ($P < 0.01$).

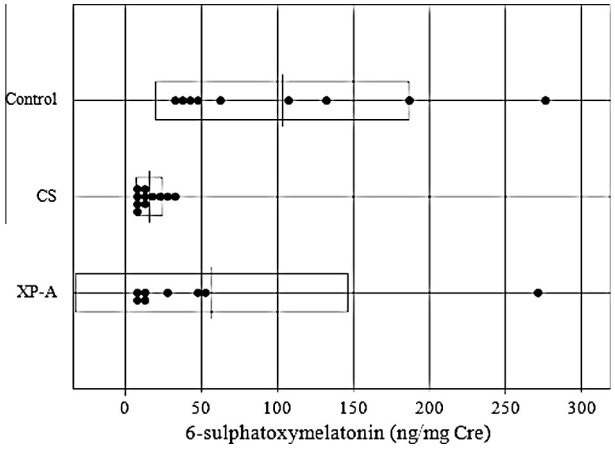

Fig. 1. Urinary secretion of 6-sulphatoxymelatonin in patients with Cockayne syndrome and xeroderma pigmentosum group A. Each vertical bar indicates the mean, while each square box denotes the area ranging from the mean minus standard deviation (SD) to the mean plus SD. Abbreviations: CS, Cockayne syndrome; XP-A, xeroderma pigmentosum group A.

In the PPN, the number of neurons that were immunoreactive for MAP2 in the CS cases was reduced significantly ($P < 0.01$) to about half of those in controls (Table 3), and astrocytes that were immunoreactive for GFAP were mildly increased (Fig. 2C). Both the numbers and percentages of AchNs that were immunoreactive for AchE were reduced significantly ($P < 0.01$) in CS cases (Table 3), whereas those that were immunoreactive for TH and CD were spared comparatively well (Fig. 2D).

4. Discussion

The sleep abnormalities and thermoregulation problems in patients with CS have not been investigated comprehensively. In the questionnaire survey, more than half of the patients with CS were said by the family to suffer from sleep disorders, such as abnormal shifts of sleep–wakefulness rhythms, frequent arousal and excitement during sleep, and daytime drowsiness (Table 1). The interrelationship of sleep problems with motor disabilities and sensory impairments was not obvious because six patients with CS and sleep disorders demonstrated various motor abilities and all had auditory and visual impairments. In addition, seven patients with CS showed thermal dysregulation, such as hypothermia, poikilothermia, and frequent fever, which were partly related to poor peripheral circulation. It is noteworthy that four patients had both sleep problems and thermal dysregulation. These data strongly suggested that circadian rhythm abnormalities should be taken into consideration as one of the major health problems in patients with CS. The investigation of sleep structures may clarify the details of sleep disorders in CS, but parents declined implementation of polysomnography.

Urinary 6-SM secretion was reduced in the patients with CS and, to a lesser extent, in the patients with XP-A. Melatonin is a neurohormone that is synthesized

Table 2
Summary of immunohistochemistry in the hypothalamus.

	Age/sex	Sleep problem	Thermal dysregulation	Cause of death	Brain weight	Tyrosine hydroxylase Supraoptic and paraventricular nuclei	Ventromedial nucleus	Vasopressin Lateral area	Orexin A Ventromedial nucleus
1	7 yrs/female	(−)	Hypothermia	Pneumonia	260 g	2+	1+	1+	2+
2	18 yrs/male	(−)	Hypothermia	Asthma status	400 g	2+	1+	1+	1+
3	18 yrs/male	(−)	Hypothermia	Renal failure	414 g	1+	1+	n/A	(−)
4	35 yrs/female	Insomnia	Frequent fever	Neuroleptic malignant syndrome	810 g	2+	1+	n/A	1+

2+, 1+, and (−) denote many neurons, a few neurons, and none, respectively, and n/A means "not assessed."

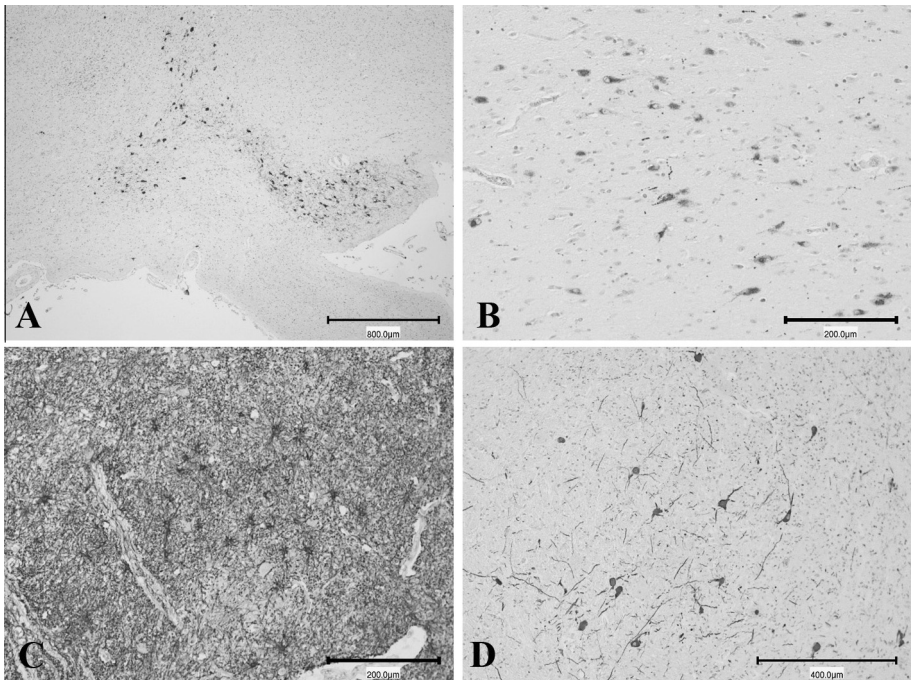

Fig. 2. Representative illustrations of immunohistochemistry in the hypothalamus and pedunculopontine tegmental nucleus (PPN). (A) An 18-year-old CS male (case 2 in Table 2) showed many neurons that were immunoreactive for tyrosine hydroxylase (TH) in the supraoptic nucleus. Bar = 800 μm. (B) Neurons that were immunoreactive for orexin A were found in the ventromedial nucleus in a 7-year-old female with CS (case 1 in Table 2). Bar = 200 μm. (C) Astrocytes that were immunoreactive for glial fibrillary acidic protein were increased in the PPN in an 18-year-old male with CS (case 2 in Table 2). Bar = 200 μm. (D) The TH-immunoreactive neurons were preserved comparatively in the PPN in an 18-year-old male with CS (case 3 in Table 2). Bar = 400 μm.

Table 3
Results of the quantitative analysis in the pedunculopontine tegmental nucleus (PPN).

		Controls (Mean (SD))	Cockayne syndrome (Mean (SD))	Mann Whitney test (p value)
Microtubule associated protein 2		243 (41)	124 (27)	<0.01
Acetylcholine esterase		68 (16)	1 (1)	<0.01
	(%)	29 (9)	1 (1)	<0.01
Tyrosine hydroxylase		25 (13)	19 (5)	ns
	(%)	10 (5)	15 (5)	ns
Calbindin-D28K		27 (17)	19 (7)	ns
	(%)	11 (6)	16 (4)	ns

Abbreviations: SD, standard deviation; ns, not significant.

in the pineal gland during the night. Pineal melatonin is important for the regulation of human circadian rhythms of sleep–wakefulness, neuroendocrine secretion, and body temperature regulation [6]. Abnormal melatonin secretion may be involved in various sleep disorders, such as shifted bedtimes and/or rising times in patients 3 and 7, and frequent arousal during sleep in patient 8 [11]. Excitement in sleep in patients 9 and 10 may also reflect frequent arousal during sleep. Accordingly, the significant reduction of urinary 6-SM secretion in the patients with CS matched the frequent occurrence of sleep disorders, which the questionnaire analysis clarified. Because melatonin secretion gradually declines with age [6], the significant reduction in urinary 6-SM secretion may be a sign of advanced aging, which has been speculated to occur in patients with CS. However, sleep disorders have rarely been reported in other progeroid syndromes, such as Hutchinson–Gilford progeroid syndrome and Werner's syndrome [12]. In addition, a reduction in urinary 6-SM secretion seemed to be more predominant in the patients with CS than in the patients with XP-A. Recently, urinary 6-SM secretion has been examined in children with developmental disorders, including autistic disorders and attention-deficit/hyperactivity disorder [13–15], although the results were heterogeneous. In this preliminary analysis, a 24-h sampling of urine was not done, and the determination of diurnal fluctuations of 6-SM secretion will be a

clue for the further clarification of disturbed melatonin metabolism in patients with CS. The detailed pathological analysis in the suprachiasmatic nucleus and the pineal gland, which was not enforceable in this analysis, may also facilitate the elucidation of pathogenesis of disturbed metabolism of melatonin.

In CS cases, the AchNs were severely reduced in the NbM, although the hypothalamic nuclei that were adjacent to the NbM were spared. A selective loss of AchNs was also found in the PPN. Clusters of neurons in the PPN project to the thalamus to trigger thalamocortical rhythms, and the descending projections from the PPN are directed to the pontine and medullary reticular formation to modulate muscle tone and locomotion [16]. The PPN is affected in Parkinson's disease and parkinsonian diseases, such as progressive supranuclear palsy, and multiple system atrophy may have an important role in gait impairments in these disorders [17]. Recently, the PPN has been proposed as a potential target for the treatment of axial symptoms in Parkinson's disease [18]. We have examined the lesions of AchNs in the PPN and/or NbM in autopsy cases of various developmental disorders. Cases with perinatal brain damage showed a reduced percentage of AchNs with a compensatory increased percentage of catecholamine neurons [19]. AchNs have been shown to be reduced in the PPNs of patients with Prader–Willi syndrome, although AchNs in the NbM were relatively well preserved [20]. The number of neurons themselves and the AchNs was reduced in cases with a history of West syndrome, while cases of dentatorubral pallidoluysan atrophy showed a decrease in the number and percentages of AchNs [21]. In this analysis, we found the similar reduction of AchNs in both the NbM and PPN in the CS brains with that in XP-A [7]. In contrast, catecholamine neurons that were immunoreactive for TH were preserved comparatively well in CS cases (Table 3 and Fig. 2D), and they were reduced in XP-A cases. Because the PPN plays a crucial role in modulating wakefulness and rapid eye movement sleep [22,23], a selective reduction of AchNs in the PPN may be involved in sleep disorders in patients with CS. It is possible that the selective reduction of AchNs was related to intellectual impairment and/or muscle tone abnormalities.

Melatonin has been used as a primary circadian regulator in children with various developmental disorders, including autism spectrum disorders and cerebral palsy, and improvements in sleep problems with minimal side effects have been confirmed in some subjects [24,25]. Because patients with CS showed reduced urinary secretion of 6-SM, irrespective of visual impairments, melatonin may possibly ameliorate their sleep problems. Recently, donepezil, a selective inhibitor of AChE, being used for the treatment of Alzheimer's disease, has been tested in Down syndrome [26]. In Japan, the standardized scores of disability increased in patients with Down syndrome after treatment with donepezil in a 24-week randomized and double-blind trial [27]. In addition, donepezil was reported to improve the disturbances of rapid eye movement sleep in five patients with autism spectrum disorder [28]. Since the complex pathomechanisms are involved in sleep disorders, it is difficult to conclude that the disturbed metabolism of melatonin and/or the selective reduction of AchNs are the major causes of sleep disorders in CS. However, the reduction of AchNs in both the NbM and the PPN may indicate the possibility that donepezil might be another therapeutic tool for sleep disorders in patients with CS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.braindev.2014.01.004.

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